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## **Protective signaling by activated protein C is mechanistically linked to protein C activation on endothelial cells**

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**Abstract:** Activated protein C (APC) has endothelial barrier protective effects that require binding to endothelial protein C receptor (EPCR) and cleavage of protease activated receptor-1 (PAR1) and that may play a role in the anti-inflammatory action of APC. In this study we investigated whether protein C (PC) activation by thrombin on the endothelial cell surface may be linked to efficient protective signaling. To minimize direct thrombin effects on endothelial permeability we used the anticoagulant double mutant thrombin W215A/E217A (WE). Activation of PC by WE on the endothelial cell surface generated APC with high barrier protective activity. Comparable barrier protective effects by exogenous APC required a 4-fold higher concentration of APC. To demonstrate conclusively that protective effects in the presence of WE are mediated by APC generation and not direct signaling by WE, we used a PC variant with a substitution of the active site serine with alanine (PC S360A). Barrier protective effects of a low concentration of exogenous APC were blocked by both wildtype PC and PC S360A, consistent with their expected role as competitive inhibitors for APC binding to EPCR. WE induced protective signaling only in the presence of wild type PC but not PC S360A and PAR1 cleavage was required for these protective effects. These data demonstrate that the endogenous PC activation pathway on the endothelial cell surface is mechanistically linked to PAR1-dependent autocrine barrier protective signaling by the generated APC. WE may have powerful protective effects in systemic inflammation through signaling by the endogenously generated APC.

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**Mechanisms of Signal Transduction:  
Protective signaling by activated protein C  
is mechanistically linked to protein C  
activation on endothelial cells**

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**PROTECTIVE SIGNALING BY ACTIVATED PROTEIN C IS  
MECHANISTICALLY LINKED TO PROTEIN C ACTIVATION ON  
ENDOTHELIAL CELLS\***

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Running Title: Protective signaling linked to protein C activation

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Activated protein C (APC) has endothelial barrier protective effects that require binding to endothelial protein C receptor (EPCR) and cleavage of protease activated receptor-1 (PAR1) and that may play a role in the anti-inflammatory action of APC. In this study we investigated whether protein C (PC) activation by thrombin on the endothelial cell surface may be linked to efficient protective signaling. To minimize direct thrombin effects on endothelial permeability we used the anticoagulant double mutant thrombin W215A/E217A (WE). Activation of PC by WE on the endothelial cell surface generated APC with high barrier protective activity. Comparable barrier protective effects by exogenous APC required a four fold higher concentration of APC. To demonstrate conclusively that protective effects in the presence of WE are mediated by APC generation and not direct signaling by WE, we used a PC variant with a substitution of the active site serine with alanine (PC S360A). Barrier protective effects of a low concentration of exogenous APC were blocked by both wildtype PC and PC S360A, consistent with their expected role as competitive inhibitors for APC binding to EPCR. WE induced protective signaling only in the presence of wildtype PC but not PC S360A and PAR1 cleavage was required for these protective effects. These data demonstrate that the endogenous PC activation pathway on the endothelial cell

surface is mechanistically linked to PAR1-dependent autocrine barrier protective signaling by the generated APC. WE may have powerful protective effects in systemic inflammation through signaling by the endogenously generated APC.

Thrombin plays a key role in the blood coagulation system because it not only activates platelets and clots fibrinogen, but also initiates the anticoagulant protein C (PC)<sup>1</sup> pathway. When thrombin binds to its receptor thrombomodulin on the surface of endothelial cells it loses its procoagulant functions and generates activated PC (APC) (1,2). Endothelial PC receptor (EPCR) binds both PC and APC and activation of EPCR-bound PC by the thrombin-thrombomodulin complex is enhanced (3). The generated APC in turn degrades cofactors Va and VIIIa that are required for efficient prothrombin activation, providing critical negative feedback regulation of thrombin generation. Intravenous infusion of recombinant APC reduces mortality in patients with severe sepsis (4) and current evidence indicates that APC has protective effects in systemic inflammation that are at least in part independent from its role as an anticoagulant.

A number of studies have indicated that exogenous APC can regulate cellular functions of endothelial cells, including gene expression (5-8), survival (9,10), proliferation (11), and barrier permeability (12-14). It is likely that many, if not all of these effects, require binding of APC to EPCR and activation of protease-

activated receptors (PARs) on the endothelial surface. Specifically activation of the thrombin receptor PAR1 is required for APC signaling in endothelial cells (7-10,13) and the EPCR-APC-PAR1 signaling cascade has been implicated in mediating neuroprotective effects of APC *in vivo* (9,15).

Compared to thrombin, APC is a very inefficient PAR1 activator and recent studies indicate that some PAR1 dependent responses of endothelial cells such as gene induction and barrier disruption require  $\sim 10^4$ -fold higher concentrations of APC than thrombin (13,16). On the other hand, our previous results demonstrate that low concentrations of APC ( $\sim 1$  nM) have powerful PAR1-dependent endothelial barrier protective effects that can be recapitulated by incubation with very low ( $\sim 40$  pM) but not higher concentrations of thrombin (13), raising the possibility that APC-PAR1 signaling may protect in systemic inflammation precisely because APC is a relatively inefficient PAR1 activator. Whereas intravenous infusion of APC has protective effects that are not entirely explained by its anticoagulant function, it is currently unknown whether the endogenously generated APC has an anti-inflammatory or other protective role in addition to its well established function as an antithrombotic agent. How can activation of the prototypical thrombin receptor PAR1 on endothelial cells by endogenously generated APC be relevant if the generation of APC is thrombin dependent and thrombin is a much more efficient PAR1 activator? Compared to APC the half-life of thrombin in the circulation is very short (17) and one possibility is that APC acts on endothelial cells that are not exposed to thrombin and are thus not involved in the activation of PC. However, the APC concentration in normal plasma is only 20-40 pM (18,19) and this APC would have to compete for EPCR binding with PC which circulates in plasma at  $\sim 80$  nM (20,21) in order to induce endothelial cell signaling. Although at sites in close proximity to ongoing clotting higher local APC concentrations may be achieved while PC is depleted, an intriguing other possibility is that autocrine APC signaling is directly coupled to the process of zymogen activation, especially given that EPCR binding supports both PC activation and APC signaling. Accordingly, we

set out in the present study to determine whether protective signaling by endogenously generated APC is enhanced compared with exogenous APC. We used a thrombin variant with higher relative specificity for PC activation to demonstrate that the endogenous PC activation pathway is indeed linked to efficient PAR1-dependent barrier protective signaling, indicating that the generated APC can be directly channeled into the protective signaling pathway.

## MATERIALS AND METHODS

*Agonists, inhibitors, and antibodies* - Human thrombin and the specific agonist peptide for PAR2 (SLIGRL) were as described (7,22,23) and human plasma derived PC, APC, and factor Xa were from Haematologic Technologies (Essex Junction, VT). Mutant recombinant human thrombin W215A/E217A (WE) was provided by Dr. Di Cera (24) and the active site concentrations of both wildtype thrombin and WE were determined by titration with hirudin using S-2238 (diaPharma, West Chester, OH) as the amidolytic substrate. Recombinant human wildtype PC and PC S360A were expressed, activated, purified, and characterized as described previously (25). The PC preparations were free of detectable APC ( $<0.1\%$ ) and thrombin ( $<0.04$  pM in 100 nM PC) as determined by amidolytic assays. PAR1 cleavage-blocking monoclonal antibodies ATAP2 and WEDE15 have been characterized previously and were used in combination at 10  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$ , respectively (7,22,26). The activity-blocking C1 anti-PC monoclonal antibody was as described (21). Hirudin was from Calbiochem (La Jolla, CA) and recombinant nematode anticoagulant protein 5 (NAP5) (27) was kindly provided by Dr. George Vlasuk (Corvas International, San Diego, CA).

*Cell culture* - The transformed human endothelial cell line EA.hy926 (28) was provided by Dr. Cora Edgell (University of North Carolina at Chapel Hill, NC). Cells were grown to confluency in a humidified atmosphere at  $37^\circ\text{C}$  in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Omega Scientific, Tazana, CA). Human aortic endothelial cells (HAECs) were obtained from Clonetics



(Walkersville, MD) and grown according to the manufacturer's recommendations.

**APC activity assay** - APC activity was measured by adding 50  $\mu$ l conditioned medium to 100  $\mu$ l of HEPES-buffered saline containing 100 nM hirudin in order to block thrombin. 50  $\mu$ l of the APC substrate S-2366 (diaPharma) or Spectrozyme PCa (#336; American Diagnostica, Stamford, CT) was added to obtain a final substrate concentration of 0.25 mM and the rate of substrate hydrolysis was determined by analyzing the change in absorbance at 405 nm over time (mOD/min) at room temperature using a  $V_{\max}$  kinetic microplate reader (Molecular Devices, Menlo Park, CA). Xa activity was determined in control experiments using Spectrozyme Xa (American Diagnostica).

**Permeability assay** - Macromolecular monolayer permeability was analyzed in a dual chamber system using Evans blue-labeled bovine serum albumin (BSA) as described previously (13,23). Briefly, EA.hy926 cells were plated on Transwell polycarbonate membranes of 3- $\mu$ m pore size and 12-mm diameter (Costar, Corning, NY). The upper and lower chambers were filled with 500  $\mu$ l and 1500  $\mu$ l growth medium, respectively. Cells were grown for 2 days to obtain a monolayer with less well established cell-cell contacts ("subconfluent cells") or for 3-4 days to obtain a tight monolayer ("confluent cells"). Agonists were added in serum-free medium containing 0.4% BSA (Calbiochem) and permeability was assayed 3 h later using 0.67 mg/ml Evans blue (Sigma, St. Louis, MO) diluted in growth medium containing 4% BSA. Fresh growth medium was added to the lower chamber and the medium in the upper chamber was replaced with Evans blue/BSA. After 10 minutes the optical density at 650 nm was measured in a 1:3 diluted 50  $\mu$ l sample from the lower chamber. For thrombin treatment of the monolayers, medium was removed and 5 nM thrombin was added for 10 min in serum free medium followed by analysis of permeability.

**Analysis of MAP kinase activation** - Mitogen-activated protein (MAP) kinase phosphorylation was analyzed by Western blotting of whole cell lysates using an anti-phospho-extracellular signal-regulated kinase-1/2 (Erk1/2) antibody (#9101; Cell Signaling Technology, Beverly, MA) in an enhanced

chemiluminescence-based detection system as described (7,13). Staining with anti-actin (Sigma) served as loading control.

**Statistical analysis** - A two-tailed t-test was used to calculate the indicated p-values.

## RESULTS

**APC generation on the endothelial cell surface** - When EA.hy926 endothelial cells were incubated in the presence of 80 nM purified human PC and different concentrations of thrombin a dose-dependent linear increase in amidolytic activity towards a chromogenic APC substrate was observed over time in the culture medium (Fig. 1A). The amidolytic activity was identified as APC using the activity blocking monoclonal anti-PC antibody C1 (21). C1 reduced the activity of purified APC as well as the generated amidolytic activity to 10-15% of control without C1 whereas the amidolytic activity of factor Xa was not affected by C1 (results not shown). No detectable amidolytic activity was generated in the absence of either PC or cells (not shown), demonstrating cell surface-dependent APC generation in our assay. The activity of 10 nM exogenously added APC decreased only slightly under the conditions of the assay (92% of the initial amidolytic activity of 34 mOD/min after 3 h). Some APC generation was consistently observed in the absence of exogenous thrombin, even when the cell layer had been extensively washed. The rate of this APC generation increased over time and it was blocked by both hirudin and the factor Xa inhibitor NAP5 (Fig. 1A). Inhibition of factor Xa with NAP5 did not affect PC activation by exogenously added thrombin in this assay (not shown) and all subsequent experiments were performed in the presence of 1  $\mu$ M NAP5 to allow a strict control of the thrombin concentration in the system.

**Barrier protective effects in response to low-dose thrombin in the presence and absence of PC** - We have previously shown in a dual chamber system measuring albumin flux that incubation with very low concentrations of thrombin (~40 pM) can increase barrier integrity of a subconfluent layer of endothelial cells and can protect a confluent monolayer from barrier disruption by a higher dose of thrombin. This protective effect was not mediated by receptor

desensitization (13). In order to test whether endogenously generated APC can support barrier protection in response to even lower concentrations of thrombin, we incubated confluent cells with 10-40 pM thrombin in the absence and presence of PC. Consistent with our previous results, 40 pM thrombin was barrier protective, whereas a significant protective effect in response to 20 pM thrombin was found only in the presence of PC (Fig. 1B). Although these results are consistent with the hypothesis that barrier protective effects of low-dose thrombin and generated APC are additive, they also indicate that it will be difficult to compare protective effects of exogenous and endogenously generated APC in this system because the thrombin concentrations required to support APC generation are similar to the concentrations that lead to direct barrier protective effects.

*WE supports PC activation at concentrations that do not induce direct signaling* - The double mutant thrombin W215A/E217A (WE) has a higher relative specificity for thrombomodulin-dependent PC activation than for PAR1 activation compared to the wildtype in a purified system using human PC/rabbit thrombomodulin and a soluble PAR1 fragment corresponding to the extracellular portion of the receptor (24). Because relative specificities on the cell surface in the presence of cofactors could be different, PC activation and PAR1 dependent signaling were analyzed on the endothelial cells. In this system, WE was almost 100-fold less active than wildtype thrombin for PC activation (Fig. 2A). However, the PAR1-dependent induction of MAP kinase phosphorylation required more than 1000-fold higher concentrations of the thrombin mutant (Fig. 2B). Thus, 2-10 nM WE lead to significant APC generation without directly inducing PAR1-dependent signaling. As shown in Fig. 2C, incubation in the presence of up to 16 nM WE did indeed not lead to barrier protective effects in the dual chamber system. These results suggest that the mutant thrombin may allow the analysis of protective signaling by endogenously generated APC in the absence of direct thrombin signaling.

*Efficient barrier protective signaling in response to WE in the presence of PC* - Cells

were incubated with a series of different concentrations of either WE in the presence of 80 nM PC or exogenous APC. After 3 h, the APC concentration in the cell medium and barrier integrity of the monolayer were measured (Fig. 3). The lowest concentration of WE that induced a significant barrier protective response in the presence of PC was 2 nM and 1.3 nM APC were generated under these conditions (Fig. 3D). For a similarly effective response 5 nM exogenous APC were required. Thus, based upon the amount of APC present after the 3 h incubation, protective signaling by WE in the presence of PC is about four times more efficient compared to exogenous APC. Importantly, APC is generated from PC at a constant rate during the incubation period while exogenous APC loses some activity. Therefore, the differential between exogenous and endogenously generated APC is at least eight-fold when the average APC concentrations during incubation are compared. We used human aortic endothelial cells (HAECs) in order to confirm that these results are not a specific property of the endothelial EA.hy926 cell line. These cells formed a tight and thrombin-responsive monolayer in our dual chamber system and exogenous APC was barrier protective at concentrations of 2 nM and higher. HAECs supported PC activation at a somewhat lower rate (~20%) compared to EA.hy926 cells, presumably because thrombomodulin expression is lower. Barrier protective signaling in response to 2 nM exogenous APC and 2 nM WE in the presence of 80 nM PC was comparably effective (Fig. 4). WE in the absence of PC had no effect. Only 0.2 nM APC were generated on HAECs during the 3 h incubation with 2 nM WE/80 nM PC. Thus, the results are consistent with those shown in Fig. 3 and they demonstrate that enhanced signaling by endogenously generated APC is not an anomalous property specific for the EA.hy926 cell line. Taken together, these data suggest that the endogenous PC activation pathway is indeed linked to efficient protective signaling. However, additional control experiments are required to rule out two alternative explanations that involve direct signaling by WE in the presence of PC: (i) Direct protective signaling by WE may be enhanced in the presence of the substrate PC independent from APC generation and (ii) sub-threshold

direct signaling by WE may potentiate signaling by APC.

*Barrier enhancement by APC is mechanistically coupled to the endogenous PC activation pathway* - Recombinant wildtype PC and a PC variant with a substitution of the active site serine with alanine (PC S360A) were used to address these possibilities. Control experiments demonstrated that after activation of the zymogens, recombinant wildtype APC was similarly effective as plasma APC in our permeability system and, as expected, the proteolytically inactive APC S360A was ineffective (Fig. 5). Cells were incubated without PC (control), with wildtype PC, or with PC S360A, in the presence or absence of WE (4 nM) and exogenous APC as indicated in Fig. 6. A low concentration of exogenous APC (3.3 nM) was used and barrier protective effects of exogenous APC were blocked by both wildtype PC and PC S360A, consistent with the expected role of zymogen PC as a competitive inhibitor for APC binding to EPCR. Incubation with exogenous APC in the presence of PC S360A actually led to a slight increase of permeability that barely reached statistical significance. WE induced protective signaling only in the presence of wildtype PC but not PC S360A, demonstrating that WE does not lead to barrier enhancement in the presence of the substrate PC unless proteolytically active APC can be generated. Finally, there was no protective effect by the combination of 3.3 nM exogenous APC and WE in the presence of PC S360A. Incubation with WE in the presence of wildtype PC led to the generation of only 0.9 nM APC and the barrier protective effects were therefore not mediated by additive direct effects of WE and APC. Taken together, these data conclusively demonstrate that efficient barrier enhancement by APC is indeed mechanistically coupled to the endogenous PC activation pathway.

*PAR1 cleavage is required for barrier protective signaling by endogenously generated APC* - Preincubation with cleavage blocking antibodies against PAR1 had no effect on APC generation but blocked barrier protective effects of the endogenously generated APC (Fig. 7). Hyperpermeability in response to the PAR1 agonist peptide was analyzed in these experiments because in contrast to thrombin the

cleavage independent PAR1 agonist peptide can disrupt barrier integrity in the presence of the blocking antibodies (13). Thus, barrier protective responses not only to exogenous APC but also to endogenously generated APC are dependent on cleavage of PAR1.

## DISCUSSION

Using a thrombin variant with a relative specificity that favors PC activation we demonstrate that the endogenous PC activation pathway on the endothelial cell surface is linked to PAR1 cleavage dependent autocrine barrier protective signaling by the generated APC. When APC is generated endogenously, the concentration of solution phase APC close to the endothelial cell surface carrying the thrombin-thrombomodulin complex is expected to be higher than the average concentration measured in the cell medium. Is it possible that the elevated local concentration is the reason why signaling by endogenously generated APC is enhanced? Barrier protection was obtained in our system when 1.3 nM APC were generated during the 3 h incubation (Fig. 3). The volume in the upper chamber is 500  $\mu$ l and thus  $3.9 \times 10^{11}$  molecules APC were generated. About 50,000 cells constitute the cell layer on the transwell membrane (1  $\text{cm}^2$ ). Because the rate of APC generation is constant over time (Fig. 1) it can be calculated from these numbers that one cell generates APC in our system at a rate of about 700 molecules/second. Francis and Palsson have developed mathematical models of diffusion for protein molecules secreted from cells and the maximal local concentration of solution phase APC directly above the cell surface at this rate of generation can be estimated to be on the order of only 0.1-0.5 nM higher than the average concentration in the supernatant (29), still much lower than the concentration of exogenous APC (5 nM) that was required for a similarly efficient protective response. In addition, solution phase APC generated endogenously would have to compete with 80 nM zymogen PC for EPCR binding in order to induce signaling. Therefore, our results cannot be explained by effects of the local concentration and we conclude that protective signaling by APC is mechanistically linked to PC activation.

The  $K_d$  for the interaction of both PC and APC with EPCR is about 30 nM (30,31). Thus, at the PC plasma concentration of ~80 nM a large fraction of the endothelial cell surface EPCR is expected to be PC-bound. EPCR-PC is activated by thrombomodulin-thrombin and our data indicate that the generated APC can activate PAR1 while still bound to EPCR. Thus, at least part of the endogenously generated APC can be directly channeled into the PAR1-dependent signaling pathway. Previous data demonstrating that PAR1 signaling by EPCR-bound exogenous APC is rapidly induced (32) and that the half-life of EPCR-bound PC as analyzed by surface plasmon resonance is approximately nine seconds (33) are consistent with this conclusion.

Could it be that the PC pathway and not thrombin may emerge as the relevant activator of endothelial cell PAR1 in response to low thrombin concentrations? Thrombin activity *in vivo* is rigorously controlled to prevent excessive platelet activation/fibrin formation. It is difficult to estimate thrombin concentrations that may be present at the endothelial cell surface *in vivo*, but a series of studies in PAR-deficient mice suggests that thrombin is generated during hemostasis and thrombosis at concentrations just above threshold levels for PAR activation: In mouse platelets PAR4 cleavage is required for thrombin signaling and PAR3 acts as a non-signaling cofactor that recruits thrombin and decreases the concentration required for half maximal signaling between 6- and 15-fold (34). However, PAR3- and PAR4-deficient mice showed similar degrees of bleeding time prolongation and protection in thrombosis models, indicating that an about 10-fold decrease in platelet responsiveness to thrombin has the same effect as complete unresponsiveness (35,36). In our *in vitro* experiments the lowest concentrations of wildtype thrombin that supported detectable PC activation and direct PAR1-dependent signaling were similar (~20 pM). However, it is possible that *in vivo* low concentrations of thrombin preferentially lead to PC activation and PAR1-dependent protective signaling by the endogenously generated APC. Thrombin binding to thrombomodulin blocks thrombin's PAR1-interactive exosite I and blood flow could affect the relative efficiency of thrombin for thrombomodulin binding versus

PAR1 cleavage. In addition, thrombin inhibitors and substrates, foremost fibrinogen, that occupy exosite I of thrombin are present in plasma at a high concentration and may shift thrombin responses from PAR1 towards PC pathway activation.  $\text{Na}^+$  binding to thrombin is required for efficient cleavage of PAR1 whereas PC activation by thrombin proceeds relatively normal in the presence of low  $\text{Na}^+$  concentrations (37). The  $\text{Na}^+$  concentration close to thrombus formation may be relatively low (38), again potentially favoring PC relative to PAR1 activation by thrombin in the endothelial microenvironment. The concept that thrombin may be able to initiate protective PC pathway signaling without activating PARs is also supported by *in vivo* evidence. Infusion of a low concentration of wildtype thrombin leads to activation of the PC pathway and blocks the lethal inflammatory response to endotoxin in animal models without eliciting platelet activation, a highly sensitive PAR-dependent response (39,40).

In a recent study, no difference in PAR1 cleavage was observed in response to a range of thrombin concentrations in the presence and absence of PC, indicating that locally generated APC did not contribute to PAR1 cleavage beyond that effected by thrombin (16). In these experiments cleavage of an overexpressed tagged PAR1 construct in transiently transfected endothelial cells was analyzed. It is possible that compared to the endogenous receptor the overexpressed PAR1 is less susceptible to cleavage by EPCR-APC. PAR1 cleavage by APC is restricted by the coreceptor requirement for EPCR whereas thrombin is expected to directly bind and cleave the overexpressed PAR1, even on cells with a very high level of the overexpressed receptor. In addition, in this study fairly high thrombin concentrations (>100 pM) were required to generate detectable conversion of PC to APC, suggesting that thrombomodulin and/or EPCR expression might have been relatively low, again favoring PAR1 cleavage by thrombin compared to APC.

Our findings raise the possibility that through endogenous APC generation the infusion of WE may induce more powerful protective effects in patients with sepsis than exogenous APC. Previous data from a baboon



model indicate that WE infusion can be antithrombotic without detectable prothrombotic activity or bleeding complications (41), suggesting that WE can be safely administered *in vivo*. Protective signaling in response to WE would be expected to depend on an intact capacity to activate PC on endothelial cells. Immunohistochemical studies have demonstrated that both thrombomodulin and EPCR expression on endothelial cells in purpuric skin lesions is reduced in children with meningococcal sepsis (42). Expression levels were variable and compared to thrombomodulin the loss of EPCR was on average less pronounced, suggesting that even endothelial cells with a strongly downregulated ability to endogenously activate PC may still respond to the EPCR-dependent protective signaling by exogenous APC. Consistent with these results, Liaw et al. have recently shown that the PC level and the ability to generate APC vary widely and independently in adult patients with severe sepsis (43). Therapy in patients with sepsis could be tailored to different subgroups based upon the PC level in plasma and the capacity for PC activation as follows: (i) Patients with a relatively normal capacity to activate PC might benefit from WE infusion to trigger powerful barrier protective effects through the endogenous PC activation pathway. Zymogen PC could be substituted in the subgroup of patients with low PC levels. (ii) On the other hand, patients with more severe disease and lower PC activation capacity may be more likely to respond to exogenous APC. Clinical studies suggest that only patients with severe sepsis and a high but not low risk of death respond favorably to therapy with exogenous APC (4,44). At least part of the reason for this finding may be that patients with a high risk of death are also expected to have on average lower levels of circulating PC and less competition for

EPCR binding. A higher dose of APC may be required for protective effects in patients with relatively high PC levels and infusion of a variant APC with reduced anticoagulant but normal anti-inflammatory activity (25) may help prevent hemorrhagic complications in these patients. In addition, especially in this group of patients a variant of APC with increased affinity to EPCR would be expected to be beneficial.

It is difficult to measure the capacity for APC generation in patients. In the study by Liaw et al. the ability to activate PC was estimated in different patients by measuring F1+2 (a marker for thrombin generation) and APC levels. A low APC level despite elevated F1+2 levels suggested that the ability to activate PC is impaired (43). However, the plasma level of APC and/or F1+2 is dependent not only on the generation but also on clearance rates of these markers, which may vary in different patients. In addition, the half-life of thrombin in the circulation is very short (17) and it is important to keep in mind that an estimate of APC generation capacity based upon these markers applies only for endothelial cells where sufficient concentrations of thrombin are present in the microenvironment to actually support PC activation. It is therefore possible that WE infusion can support PC activation even in patients with elevated F1+2 levels and a low APC level by targeting endothelial cells that are not exposed to the thrombin generated endogenously.

In conclusion, our finding that powerful autocrine barrier protective signaling is mechanistically linked to the endogenous PC activation pathway has important implications for novel approaches to treat systemic inflammation in patients.

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#### FOOTNOTES

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<sup>1</sup> The abbreviations used are: APC, activated protein C; BSA, bovine serum albumin; EPCR, endothelial protein C receptor; PAR, protease activated receptor; PC, protein C; WE, double mutant thrombin W215A/E217A

#### FIGURE LEGENDS

**Fig 1. APC generation on the endothelial cell surface and barrier protective signaling.** (A) APC generation. EA.hy926 cells were grown to confluency in 12-well plates followed by three washes with 1 ml serum free medium. The cells were incubated at 37°C in the presence of 80 nM PC in the absence or presence of the indicated concentrations of thrombin. Where indicated, the Xa inhibitor NAP5 (1 µM; open triangles) or the thrombin inhibitor hirudin (100 nM; open squares) were present. At the indicated time points amidolytic APC activity was determined in the cell medium using the chromogenic substrate S-2366 in the presence of 100 nM hirudin. A typical experiment of 4 is shown. (B) Barrier protective signaling. Confluent cells in a dual chamber system were incubated for 3 h in serum-free growth medium with the indicated concentrations of thrombin in the absence (gray bars) or presence (black bars) of 80 nM PC in

both the upper and lower chamber. Permeability was tested using Evans blue-labeled albumin before (open bars in each group) and after (closed bars) an additional 10 min incubation with 5 nM thrombin. Means $\pm$ SEM are shown (n=6, \*p<0.05 compared to no thrombin). P-values comparing results in the absence and presence of PC are indicated (*ns*=not significant).

**Fig 2. PC activation and PAR1-dependent signaling by wildtype thrombin and thrombin W215A/E217A (WE).** (A) PC activation. APC activity generated upon 90 min incubation of confluent EA.hy926 cells in 12-well plates with 80 nM PC and the indicated concentrations of either wildtype thrombin (closed circles) or WE (open circles) was determined using the chromogenic substrate S-2366 (means $\pm$ SEM, n=2). (B) PAR1 dependent signaling. Erk1/2 phosphorylation compared to control was analyzed after a 5 min incubation with the indicated concentrations of either wildtype thrombin (closed circles) or WE (open circles) (means $\pm$ SEM, n=3). (C) Confluent cells in the dual chamber system were incubated for 3 h with the indicated concentrations of WE in the upper chamber. Permeability was tested before (open bars) and after an additional 10 min incubation with 5 nM thrombin (closed bars) (means $\pm$ SEM, n=4).

**Fig 3. Barrier protective signaling in response to WE in the presence of PC versus exogenous APC.** Confluent EA.hy926 cells were incubated for 3 h with either the indicated concentrations of WE + 80 nM PC or exogenous APC in the upper chamber. (A,B) Permeability was tested before (open bars) and after (closed bars) an additional 10-min incubation with 5 nM thrombin. (C) APC activity in the upper chamber after the 3 h incubation in the presence of WE + 80 nM PC (left panel) or APC (right panel) was determined using the chromogenic substrate Spectrozyme PCa. Of note, this substrate is hydrolyzed by APC slightly less efficiently than the S-2366 used in Fig. 1 and 2. Results are plotted with linear regression lines. (D) Permeability is plotted as a function of APC concentration for the endogenously generated APC (open circles) and exogenous APC (closed circles). Means $\pm$ SEM are shown (n=6, \*p<0.05 compared to control).

**Fig 4. Efficient barrier protective signaling in response to endogenous APC generation on HAECs.** Confluent human aortic endothelial cells (HAECs) were incubated for 3 h with WE in the absence and presence of PC or with exogenous APC as indicated. Permeability was tested before (open bars) and after (closed bars) an additional 10-min incubation with 5 nM thrombin. APC activity in the upper chamber after the 3 h incubation was determined using the chromogenic substrate Spectrozyme PCa. Means $\pm$ SEM are shown (n=6, \*p<0.05 compared to control).

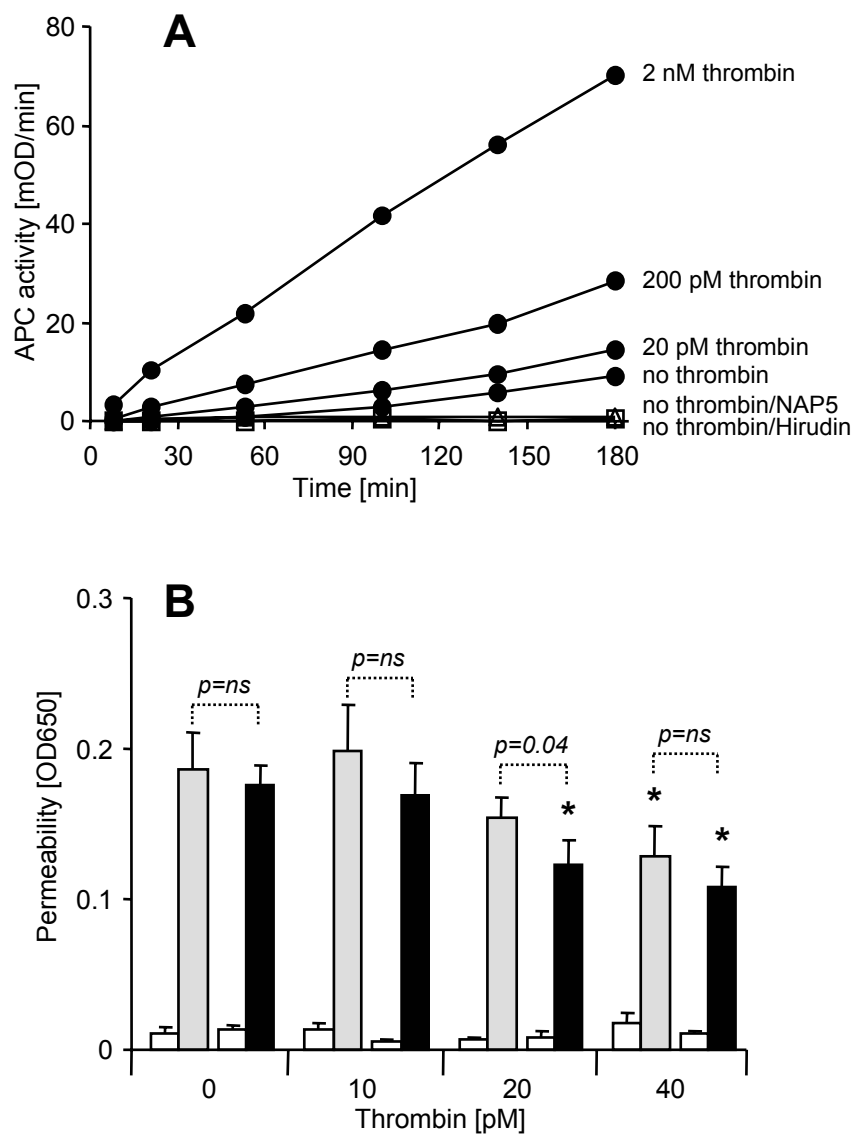
**Fig 5. Effects of recombinant wildtype APC and APC S360A on endothelial barrier integrity.** Subconfluent (upper panels) or confluent (lower panels) EA.hy926 cells were incubated for 3 h with the indicated agonists in both the upper and lower chambers at either 4 nM (left panels) or 16 nM (right panels) followed by determination of permeability. Permeability of confluent cells was tested before (open bars) and after (closed bars) an additional 10-min incubation with 5 nM thrombin. Shown are means $\pm$ SEM (n=8, \*p<0.05 compared to control).

**Fig 6. Mechanistic coupling of PC activation and barrier protective signaling by APC.** Confluent EA.hy926 cells were incubated with control or 80 nM recombinant wildtype PC

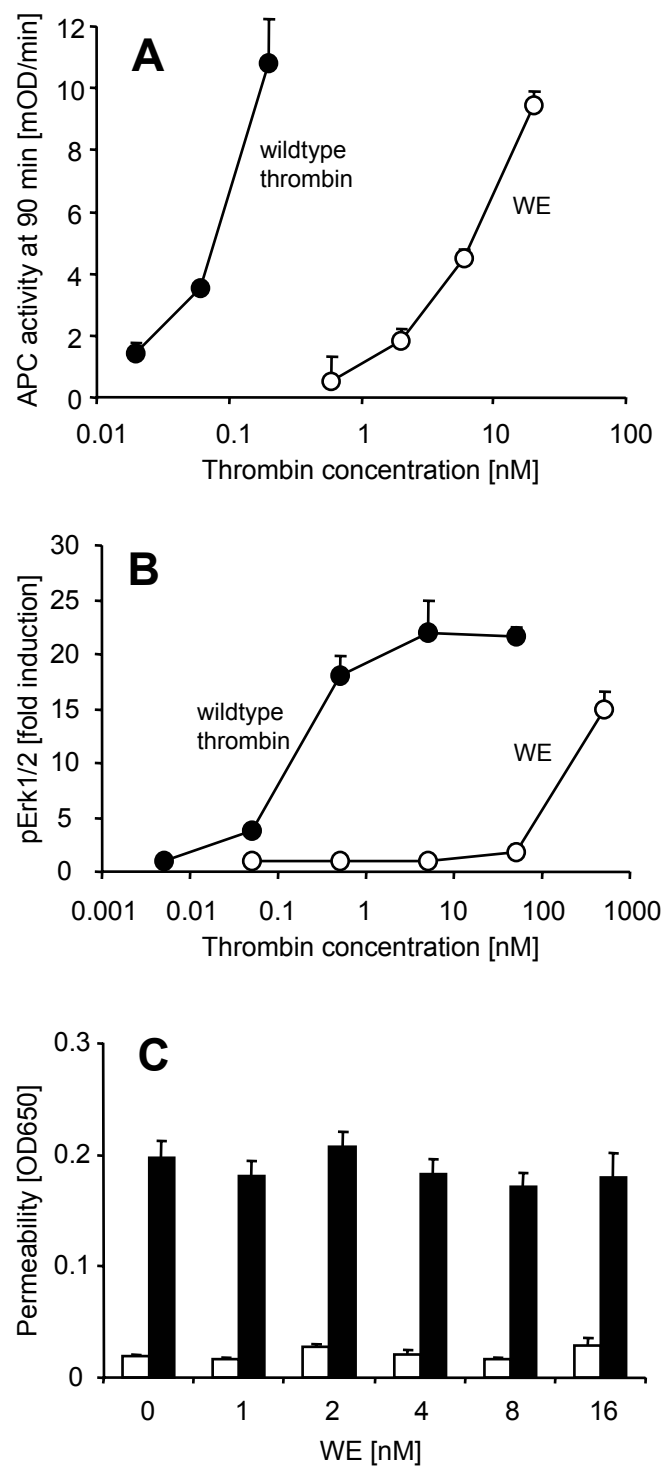


or PC S360A in the upper chamber as indicated. In each group 4 nM WE and 3.3 nM APC were also present either alone or in combination. After 3 h, APC activity in the upper chamber was determined using the chromogenic substrate Spectrozyme PCa and permeability was tested before (open bars) and after (closed bars) an additional 10 min incubation with 5 nM thrombin. Shown are means $\pm$ SEM (n=8, \*p<0.05, \*\*p<0.005 compared to no agonist).

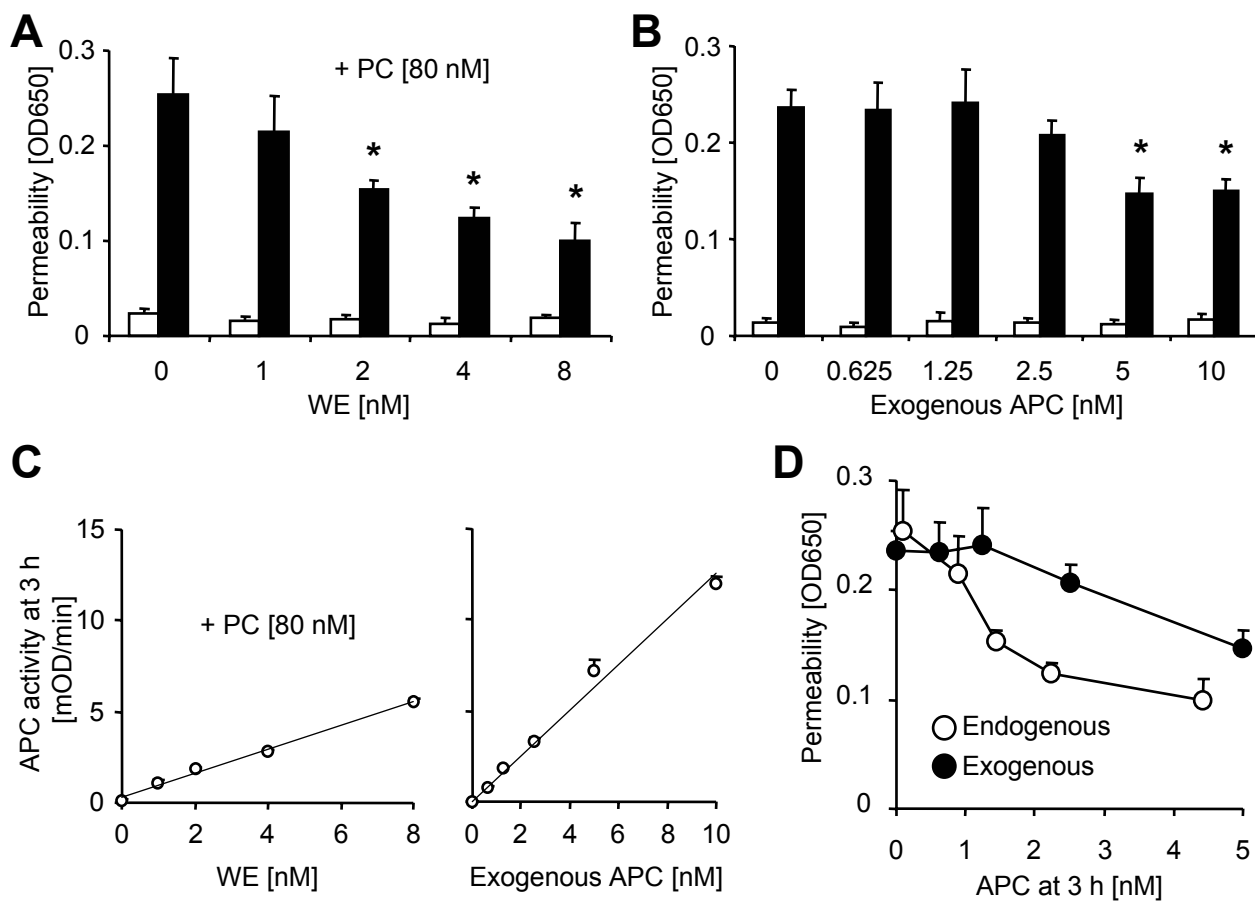
**Fig 7. Effect of cleavage-blocking anti-PAR1 on barrier protective signaling by endogenously generated APC.** Confluent EA.hy926 cells were incubated for 3 h with the indicated agonists in the upper chamber in the absence (left panel) or presence (right panel) of cleavage blocking anti-PAR1. PAR2 can induce barrier protective effects (23) and the PAR2 agonist peptide SLIGRL was used as a control. APC activity in the upper chamber was tested at the end of the 3 h incubation period using the chromogenic substrate Spectrozyme PCa. Permeability relative to no agonist control after an additional 10 min incubation with 20  $\mu$ M PAR1 agonist peptide is shown (means $\pm$ SEM, n=8, \*p<0.05, \*\*p<0.005 compared to no agonist).



**Figure 1**

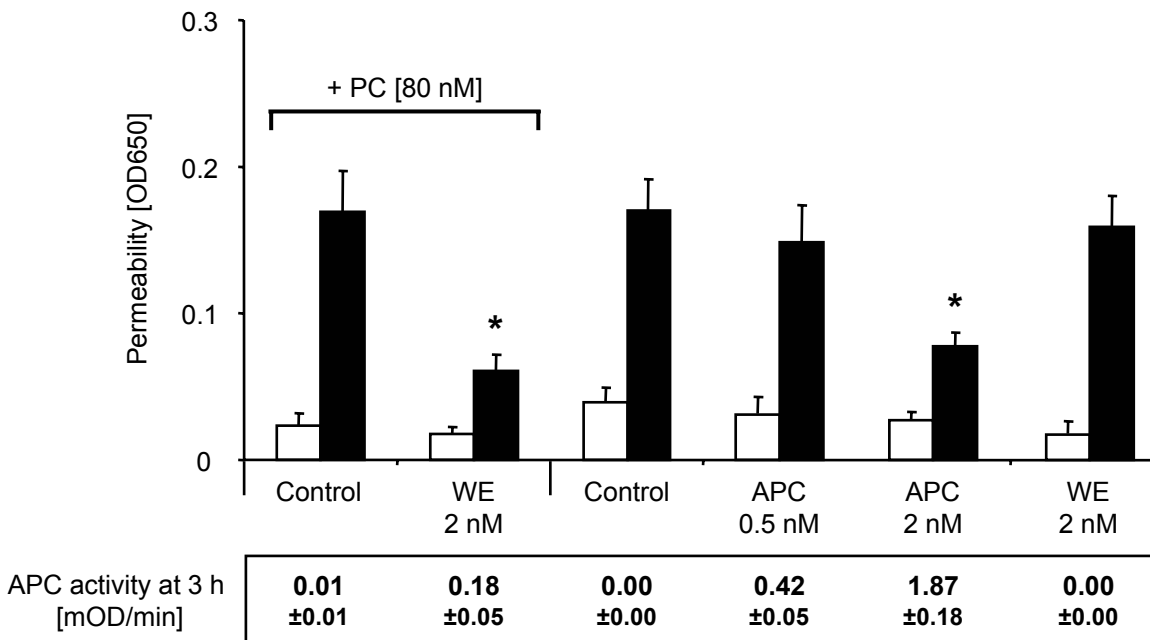


**Figure 2**

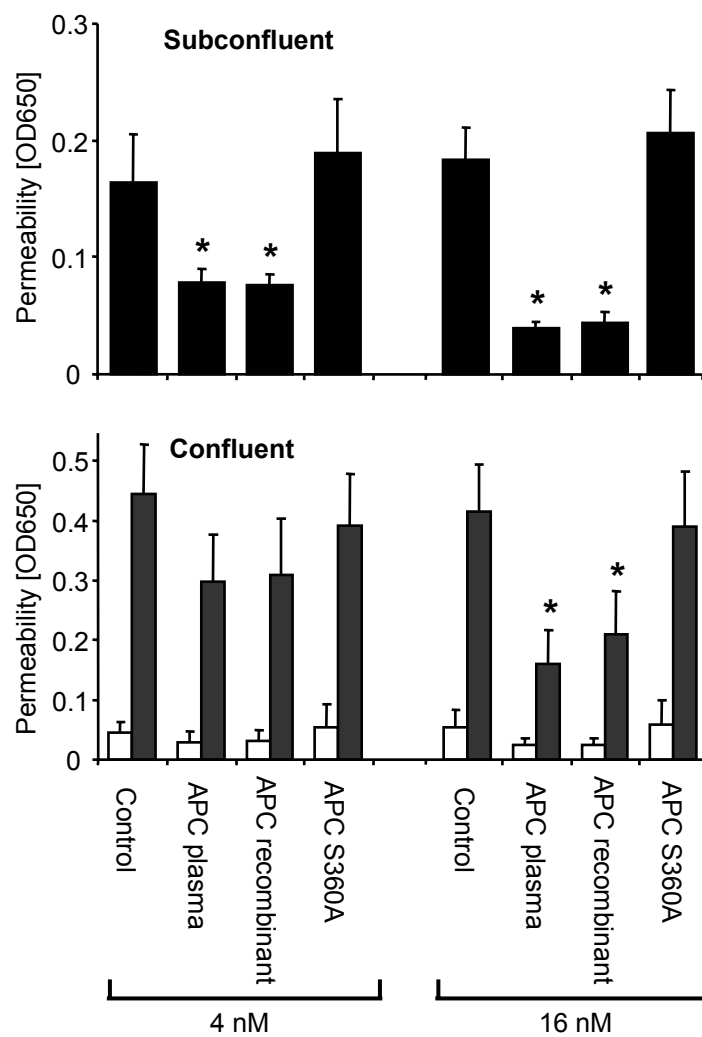


**Figure 3**

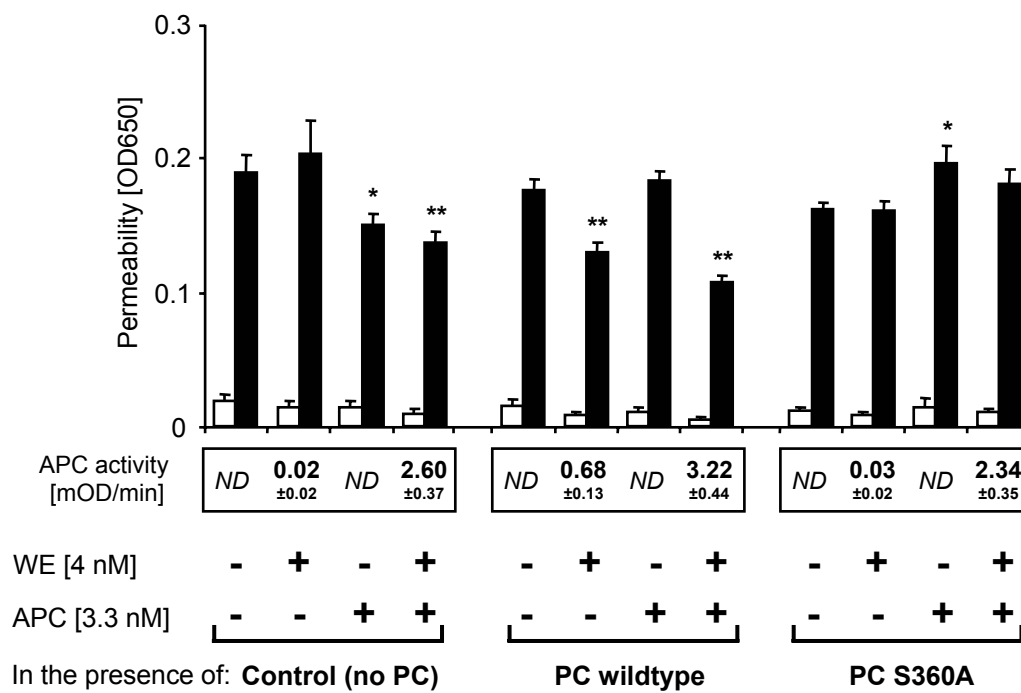




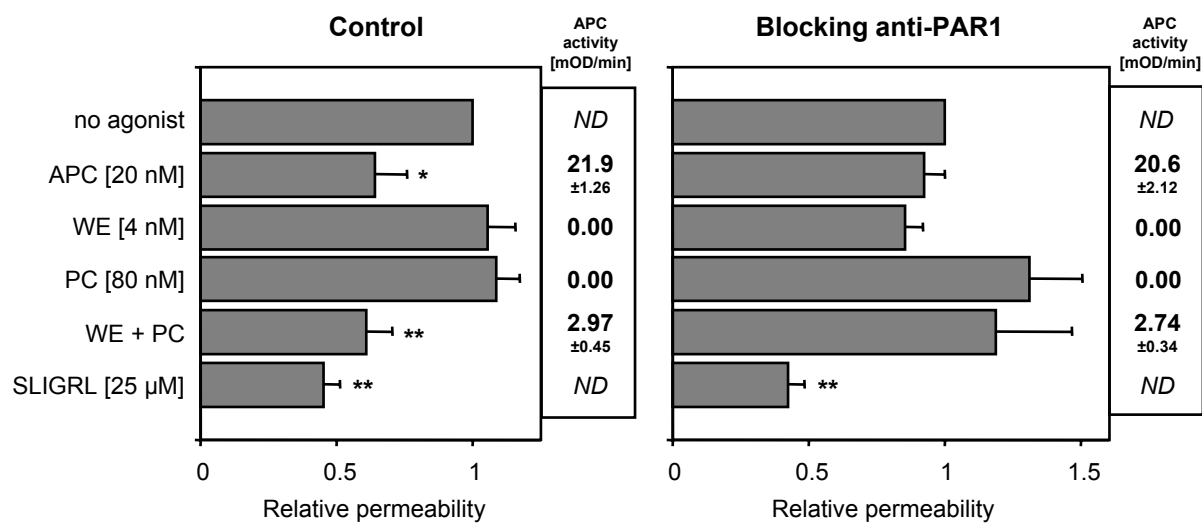
**Figure 4**



**Figure 5**



**Figure 6**



**Figure 7**